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13. ABSTRACT *(Maximum 200 Words)*

Acquired tamoxifen resistance is common in breast cancer patients with estrogen receptor positive (ER+) tumors. Growth factor signaling can provide ER positive breast cancer cells with alternative growth stimulus to that which is provided by activation of ER. A lipid-anchored docking protein SNT-1 links FGFR molecules with the Ras/MAP kinase signaling pathway. The main objective of this work is evaluation of SNT-1 as a useful target for drug development. For this purpose we cloned a phosphotyrosine binding domain (PTB) of SNT-1 in the expression vectors pTRE Ins, which provides tetracycline regulation of protein expression, and pEF6. Breast cancer cells transfected with these plasmids expressed the PTB domain, which was shown to inhibit tyrosine phosphorylation of cellular SNT-1 protein and greatly decrease MAP kinase activation in FGF-dependent conditions. These data demonstrate dominant negative properties of the PTB domain and validate it as a possible target for drug design. We also found evidence for involvement of SNT-1 independent mechanisms in FGF1 signaling in breast cancer cells and we are studying these mechanisms. We continued our efforts to crystallize the SNT-1 PTB domain and to study its possible binding to different FGF receptors.

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Table of Contents

INTRODUCTION

Acquired tamoxifen resistance is common in breast cancer patients with estrogen receptor positive (ER+) tumors. Second line hormonal therapies have limited effectiveness in patients who have failed following a response to tamoxifen treatment for metastatic disease or relapsed after receiving adjuvant tamoxifen therapy. A better understanding of the mechanisms responsible for acquired tamoxifen resistance should provide the conceptual basis for additions or modifications to current treatment regimens that will restore sensitivity or prolong the therapeutic effect of tamoxifen and other selective estrogen receptor response modifiers. Growth factor signaling can provide ER positive breast cancer cells with alternative growth stimulus to that which is provided by activation of ER. Data from the laboratory of the principal investigator indicate that FGF-1 and FGF-4 transfected MCF-7 cells become tamoxifen resistant and cross resistant to second line antihormonal agents including pure antiestrogen ICI 182,780 (Faslodex) and two aromatase inhibitors (1). An enhanced growth of FGF-transfected or FGF-treated ERα+ breast cancer cells in medium containing ICI 182,780, an antiestrogen which greatly increases the rate of ER degradation, suggests that FGF signaling completely bypasses a requirement for ERα activation for growth.

The RAS/MAPK pathway plays an important role in signaling via FGF receptors. Published work from other laboratories has indicated that a lipid-anchored docking protein called SNT-1 links FGFR molecules with the Ras/MAP kinase signaling pathway (2). It was demonstrated that FRS2 protein, a mouse analog of SNT-1 protein, also plays a role in FGF-induced phosphatidylinositol-3(PI-3) kinase activation (3). Targeted disruption of the FRS2 gene caused severe impairment in mouse development resulting in embryonic lethality, establishing the central role of FRS2 in signaling via FGFRs (3). SNT-1 protein contains a phosphotyrosine binding (PTB) domain at its amino terminus. This PTB domain binds to a juxtamembrane region of FGFR1 (4). Several proteins, including Grb2, Sos, Shp2, Crk, Gab1 bind to SNTs following FGF induced SNT phosphorylation (5,6). Some cellular fractions of atypical protein kinase C are able to bind SNT-1 regardless of its phosphorylation (7). Because FGF is a growth factor that may have paracrine as well as autocrine effects within a tumor, including effects on

angiogenesis, interference with this binding may also block the growth promotion that occurs in endothelial cells or other stromal elements. In this proposal, we intended to validate SNT-1 as a useful target for drug development (objective 1), determine if all four FGF receptors can interact with SNT-1 through homologous regions (objective 2), and determine a crystal structure of the SNT-1 PTB domain (objective 3).

BODY

Evaluation of dominant negative properties of the PTB domain of SNT-1.

In the first year of the project we encountered difficulties involving the use of the vector pCMVTag from Stratagene for the expression of the PTB domain of SNT-1 in breast cancer cells. Therefore, we used a different expression system. RT PCR generated cDNA encoding the N-terminal 140 aminoacids of the SNT-1 protein (including the myristylation signal and the phosphotyrosine binding domain) in front of an in frame C terminal c-myc epitope was cloned in the vector pEF6 (Invitrogen) which also directs the expression of a blasticidin resistance gene as a selective marker. The breast cancer cell line ML20, a derivative of the MCF-7 cell line (8) was stably transfected with this construct. Cells transfected with the empty vector were used as a negative control. Levels of expression of the SNT-1 PTB domain in blasticidin resistant populations were determined by immunofluorescence microscopy using primary antibodies (Invitrogen) against the c-myc epitope. To visualize c-myc tagged protein, we used secondary antibodies conjugated with Texas Red from Jackson ImmunoResearch Laboratories, Inc. Since the polyclonal population of PTB domain transfected cells showed mosaic expression of the protein in different cells, we selected two clones (clone 2/1 and clone 5) demonstrating uniformly high levels PTB domain expression. To confirm the expression of the recombinant SNT-1 PTB domain in the ML20 cells, we performed an immunoblot with the clone 9E10 anti-c-myc mouse monoclonal antibody from Oncogene Research Products. As it is shown at Fig. 1, a protein containing the c-myc tag is expressed only in the cells transfected with PTB-domain containing plasmid

pEF6 but not in the untransfected ML20 cells or in the cells transfected with the vector pEF6 alone.

To determine the dominant negative properties of the SNT-1 PTB domain, we characterized the effect of PTB domain overexpression on the levels of wild type SNT-1 tyrosine phosphorylation in response to FGF1 stimulation. We stripped transfected cells of estrogen by repeated changes with PRF-IMEM and incubated cells with 20 ng/ml of FGF1 in PRF-IMEM for 15 and 30 minutes. One mg of the p13Suc1–agarose (Upstate Biotechnology) captured fraction of a total cell lysate was electrophoresed in a 7.5% SDS-polyacrylamide gel and subjected to immunoblotting with the anti-phosphotyrosine monoclonal antibody, clone 4G10 from Upstate Biotechnology. As is shown at Fig. 1, there was a significant decrease of phosphorylation of the wild type SNT-1 in clone 2/1 and clone 5 compared to untransfected cell or to the cells transfected with the vector only. We confirmed that equal quantities of wild type SNT-1 protein were precipitated with the p13Suc1 agarose by reprobing the stripped membrane with FRS2 antibodies from Santa-Cruz Biotechnologies (Fig.1).

To further evaluate the dominant negative function of the PTB domain of SNT-1, we determined levels of phosphorylation of the two key proteins of FGF signaling, MAP kinase and AKT, in the clones of ML20 expressing the PTB domain. Cells were stripped of estrogen and incubated for 30 minutes in the presence of FGF1 in serum-free PRF-IMEM medium. 30 ug of total cellular lysates were used for immunoblotting with phospho specific antibodies against Thr202/Tyr204 of MAPK (Cell Signaling) and 100 μg of total cellular lysates for phospho specific Ser473 of AKT (Cell Signaling). Surprisingly, phosphorylation of both proteins in both clones was not diminished (Fig.1). To evaluate dominant negative effect of the SNT-1 PTB domain cloned in the vector pEF6 on cellular growth, we sought to determine if the presence of the SNT-1 PTB domain would result in a decrease in the number of blasticidin resistant colonies in the FGF dependent growth conditions of 10^{-7} M of ICI 182,780 and 20 ng/ml of FGF1. For this assay, we stripped ML20 cells of estrogen with 5% CCS PRF-IMEM for 3 days, transfected cells with the above mentioned plasmid or with the vector alone, and

monitored cell growth in the 3.0 μg/ml of blasticidin in each of the following conditions: PRF-IMEM + 5% FBS, PRF-IMEM+5% FBS +10^{-7} M ICI 182,780, PRF-IMEM + 5% FBS + 10^{-7} M ICI 182,780 + 20 ng/ml FGF1. Medium was changed every 3-4 days and colonies were stained and counted after 2-3 weeks. We also did not observe any growth inhibition due to the PTB domain in any of these conditions. While FGF signaling does not appear to be critical for the maintenance of MCF-7 breast cancer cells in culture, it is possible that cells with impaired FGF or NGF signaling as a result of constitutive SNT-1 PTB expression may have a selective disadvantage. Therefore, it is possible that in our stable transfection experiments we could be selecting for cells where additional mechanisms can compensate for the function of SNT-1. To explore this possibility we used a transient transfection assay to visualize a possible specific FGF-dependent inhibition of MAP kinase phosphorylation in the presence of high levels of SNT-1 PTB domain in the cell. We used reporter plasmid encoding a GFP-MAP kinase fusion protein (expression vector was kindly provided by E. Nishida, Japan) for this transient transfection experiment. The molecular weight of the fusion GFP-MAPK protein is around 80 kD which allowed us to distinguish this protein from wild type Erk1 and Erk2 with molecular weights of 42 and 44 kD. We cotransfected ML20 cells with GFP-MAPK and pEF6/PTB plasmids and after 48-72 hours of incubation determined levels of GFP-MAPK threonine/tyrosine phosphorylation in the FGF-stimulated cells. Fig. 2 demonstrates an inhibition of FGF-dependent GFP-MAPK phosphorylation in the cells transiently cotransfected with the pEF6/PTB construct compared with cells transfected with the empty vector. These results indicate the PTB domain of SNT-1 as a dominant negative molecule capable of inhibiting a key step in FGF receptor-mediated signal transduction pathways.

A possible mechanism for compensation of an inactive SNT-1 protein in FGF signaling could involve increased signaling through the ShcA adaptor protein (9,10). If this hypothesis is correct, the levels of protein ShcA phosphorylation would be expected to be increased in cells expressing the SNT-1 PTB domain to compensate for the absence of SNT-1 protein function in these cells. We induced these cells with 20 ng of FGF in the serum free PRF-IMEM for 30, 60, and 180 min, and determined the levels of Y317

tyrosine phosphorylation using anti-phospho-SHC (Y317) antibodies from Upstate Biotechnology. This experiment did show an increased level of ShcA phosphorylation in the clones stably transfected with the PTB domain of SNT-1 (Fig.3). To confirm the involvement of the protein ShcA in the FGF pathway, we intend to use dominant negative mutants to abrogate Shc signaling. We have recently received a HA tagged Shc Y239F, Y240F,Y317F triple mutant from Dr. K. Ravichandran that has a dominant negative function (11). To determine its role in the FGF signaling pathway we plan to cotransfect cells expressing the PTB domain of SNT-1 with this mutant and GFP-MAPK and check the possibility of further decreasing the residual MAPK phosphorylation that occurs in these cells upon FGF induction.

To clarify the role of the SNT-1 protein in FGF signaling and cell growth, we used a tetracycline-inducible expression system (12), which would allow us to avoid compensatory mechanisms that may occur as a result of constitutive expression of the PTB domain. For this purpose we subcloned c-myc tagged SNT-1 PTB domain into a modified tetracycline response element (TRE) vector constructed in our laboratory that utilized flanking HS4 insulator elements to reduce basal expression in the absence of tetracycline (Qu at al., manuscript in preparation). We have utilized a clone of ML20 cells designated C9 that is stably transfected with the "tet on" transactivator rtTA2 (S)-M2 (11). We cotransfected these cells with the PTB/pTRE Ins construct and a plasmid conferring zeocin resistance and individual clones selected in the presence of blasticidin and zeocin were screened. Two clones (clone 29 and clone 13) with high and homogenous doxycycline-induced c-myc epitope tag fluorescence were chosen for further characterization (Fig.4). High levels of the c-myc tagged PTB domain expression induced by incubation in the presence of 200 ng of doxycycline were confirmed by the immunoblot with monoclonal c-myc epitope antibody in both clones (Fig.5). We did not observe any detectable basal expression of the PTB domain in the absence of doxycycline.

Since MAP kinase is a critical end point in the FGF signal transduction pathway, we determined the levels of MAP kinase phosphorylation in the cellular lysates of both

clones in the presence of FGF-1 and in the presence and absence of 200 ng/ml doxycycline. As a control, we also stimulated the same cells with heregulin β1 since this signaling pathway does not involve the SNT-1 protein. We showed that in the presence of doxycycline the FGF dependent phosphorylation of MAPK is strongly inhibited compared to ML20 C9 cells or the same parental cells transfected with the empty pTRE Ins vector. There was no MAPK phosphorylation inhibition observed in the absence of doxycycline neither in the PTB/TRE clones, pTRE transfected cells, or parental ML2- C9 cells (Fig.6). We did not observe any inhibition of MAPK phosphorylation induced by heregulin when doxycycline was present in any type of the cells. These data confirm the dominant negative properties of the PTB domain in selectively inhibiting FGF signaling. They also confirm a critical role of SNT-1 protein and specify it as a potential target for drug design.

SNT-1 protein also plays a pivotal role in FGF-induced recruitment and activation of PI3-kinase. Tyrosine phosphorylation of SNT-1 leads to Grb2-mediated complex formation with the docking protein Gab1 and its tyrosine phosphorylation , resulting in the recruitment of PI3K, which in turn activates a downstream effector proteins, such as AKT (12). Despite the effect of Dox-induced SNT-1 PTB domain expression on MAPK phosphorylation, we did not observe any decrease in phosphorylation of Akt in the FGF dependent conditions in the presence or in the absence of doxycycline as determined by immunoblot with PhosphoPlus AKT (Ser473) antibody kit from Cell Signaling (Fig.7). In the coming year we intend to confirm the PTB domain overexpression does indeed inhibit the formation of the wild type SNT-1-Grb2-Gab1 complex in response to FGF addition to breast cancer cells.

Determining a crystal structure of the phosphotyrosine binding domain of SNT-1 protein.

In our model we suggested that residues 401-435 of FGFR1 would be important for the complex formation with the PTB domain of SNT-1. Recent literature data supported this suggestion. The importance of residues 412-433 of FGFR1 in the complex

formation with the PTB domain of SNT-1 was recently demonstrated by mutational analysis and coimmunoprecipitation (13). Moreover, as suggested in our model, the basic residues 418-427 were needed for this interaction. A recently published NMR analysis (13) did indeed suggest that FGFR1 residues 411-430 interact with a separate domain that is distinct from the phosphotyrosine binding site. Thus NMR data confirmed our original suggestion about the importance of the acidic groove for binding of highly conserved aminoacids in the juxtamembrane region of FGFR.

As we reported last year, the SNT-1 PTB domain (residues 11-140) was overexpressed in E.coli, and recombinant protein was purified using an N-terminal 6His-Tag. Although the protein ran as a single band in SDS PAGE, the native gel electrophoresis showed smeared bands (Fig.8, lanes 2-4), which indicated that SNT-1 molecules aggregate in the solution. To stabilize the protein in solution and to facilitate crystallization, four minimum interaction peptides containing conserved amino acid sequences of FGFR1 (amino acids 411-430, Genbank accession # M34185), FGFR2 (amino acids 412-431, Genbank accession # NM_000141), FGFR3 (amino acids 408-427, Genbank accession #M58051), and FGFR4 (amino acids 403-422, Genbank accession # Y113901) were synthesized. SNT-1 incubated with one of these peptides showed increased stability (Figure 8, lanes 5-16), particularly for PS67 and PS68 derived from FGFR4 and FGFR3, respectively.

Peptide	*Sequence*	*Receptor*
PS65	(411-430)-QMAVHKLAKSIPLRRQVTVS	FGFR-1
PS66	(412-431)-QPAVHKLTKRIPLRRQVTVS	FGFR-2
PS68	(408-427)-SPTVHKISRFPLKRQVSLES	FGFR-3
PS67	(403-422)-PATVQKLSRFPLARQFSLES	FGFR-4

We have attempted to crystallize SNT1 protein with and without the presence of one of the polypeptides derived from FGFRs. About 300 solutions have been used for initial screening of crystallization conditions by a vapor diffusion sitting-drop method. The initial protein concentration used was about 10.5 mg/ml. Most of the crystallization

drops were clear, suggesting protein concentration is much lower than the concentration that is needed to reach super-saturation. The protein concentration was then increased to about 18.5 mg/ml. After more than 1,200 conditions tested, only micro-crystals were observed. The best lead condition is with PS66 peptide present in a solution containing 0.1M tri-sodium citrate (pH 5.6), 35%(v/v) tert-butanol. We are currently expanding our screening experiment with several lead conditions.

Key research accomplishments.

- The c-myc tagged PTB domain of SNT-1 protein was overexpressed in the MCF-7 breast cancer cell line in either a constitutive or inducible manner using vectors pEF6 and pTRE Ins, respectively. The vector pTRE Ins allowed inducible overexpression of the protein at very high levels and avoidance of the potential problems of adaptation to constitutive overexpression of SNT-1 PTB with compensatory mechanisms of FGF signaling.

- Dominant negative properties of the SNT-1 PTB domain were confirmed by an inhibition of wild-type SNT-1 phosphorylation in cells stably transfected with the PTB domain and by inhibition of MAP kinase phosphorylation upon FGF stimulation in either cells transiently transfected with SNT-1 PTB vector and GFP-MAPK or in the cells expressing a doxycycline regulated form of the SNT-1 PTB protein.

- Although inducible SNT-1 PTB overexpression resulted in specific inhibition of FGF-dependent MAPK phosphorylation there was no effect on AKT phosphorylation. This suggests an alternative mechanism of PI3Kinase activation in FGF stimulated cells that is independent of the activation of Gab1.

- Our results suggest a compensatory mechanism involving increased signaling through Shc may substitute for SNT-1 function in the FGF pathway in MCF-7 cells.

- Lead conditions for crystallization of purified SNT-1 PTB domain have been determined.

Reportable outcomes

Marina Manuvakhova, Jaideep Thottassery, Susan Hays, and Francis G. Kern. Evidence for involvement of SNT-1 independent mechanisms in FGF signaling in breast cancer cells. Abstracts of the 93rd Annual Meeting of the American Association for Cancer Research. April 6-10, 2002, San-Francisco, California, p.728.

Conclusions.

At the end of the second year of our study, we have completed many of the goals listed in Task 1. It was shown that the truncated protein consisting of an N-terminal myristylation signal and the PTB domain with a deleted carboxy terminal region containing phosphotyrosine docking sites for Grb2 and SHP-2 signaling molecules can be overexpressed in breast cancer cells at very high levels in either a constitutive or regulatable manner. The truncated protein exhibits dominant negative properties by abrogating tyrosine phosphorylation of the cellular SNT-1 protein. Upon transient expression or inducible expression at high levels, the PTB domain inhibited MAPK phosphorylation, a key end point in the FGF signaling pathway. We also showed that in the cells expressing the protein in an inducible manner, phosphorylation of AKT is not affected suggesting that a separate mechanism of AKT activation exists in these cells.

Overexpression of the PTB domain by means of tetracycline regulated expression allowed us to test an effect of this protein on the anchorage dependent growth of the stably transfected ML20 cells in estrogen depleted medium and other FGF-dependent growth conditions. The use of other growth factors such as heregulin β1 that also permit growth in antiestrogen containing media will allow us to determine the specificity of the dominant negative properties of the PTB domain for FGF signaling.

To explore the pathways leading to the inhibition of MAP kinase in the cells expressing the PTB domain of SNT-1, we plan to determine if this disrupts an interaction between wild type SNT-1 and the downstream signal transduction molecules Grb2 using coimmunoprecipitation and immunoblotting assays. We also plan to study an involvement of SOS and RAS in this process. It will also be important to explore the activation state of the PI3 kinase pathway in the cells expressing the dominant negative mutant of SNT-1 protein by determining whether overexpression of the SNT-1 PTB disrupts the association of wild type SNT-1 with Gab1.

We plan to continue to study an involvement of a potential signal adapter, Shc, as an alternative way of linking FGF receptor activation to the activation of Ras protein in breast cancer cells. If Shc-dependent signal transduction is indeed involved in cellular adaptation to the effects of SNT-1 dominant negative mutants, this fact will be taken into consideration in consequent drug design approaches.

The coordinates for the NMR derived structure published by another group (13) should be available in the near future. This structure can be used for drug design. The NMR structure in essence validated our initial homology model by demonstrating an interaction of the basic region of the FGFR-1 peptide with an acidic groove that constitutes a second binding site distinct from the phosphotyrosine binding site. Consequently we are now focusing our efforts on the specificity of the interaction of SNT-1 protein with different FGF receptors. We plan to first use a Biacore apparatus to determine if a high affinity interaction of the PTB domain occurs with FGFR1, 2, 3, and 4 juxtamembrane peptides.

References

1. McLeskey, S,W., Zhang ,L., El-Ashry, D., Trock, B.J., Lopez, C.A., Kharbanda, S., Tobias, C.A., Lorant, L.A., Hannum, R.S., Dickson, R.B., and Kern, F.G. Tamoxifen-resistant FGF-transfected MCF-7 cells are cross-resistant in vivo to the antiestrogen, ICI 182,780 and two aromatase inhibitors. Clin. Cancer Res. 4:697-711, 1998.
2. Kouhara, H., Hadari, Y.R., Spivak-Kroizman,T., Schillling, J., Lax, I., and Schlessinger, J. A lipid-anchored Grb2 binding protein that links FGF receptor activation to the Ras/MAP kinase signaling pathway. Cell, 89:693-702, 1997.
3. Hadari, Y.R., Gotoh, N., Kouhara, H., Lax I., Schlessinger, J. Critical role for the docking-protein FRS2α in FGF receptor-mediated signal transduction pathways. PNAS, 98:8578-8583
4. Xu, H., Lee, K.W., and Goldfarb, M. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J. Biol.Chem., 273:17987-17990, 1998.
5. Hadari, Y.R., Kouhara, H., Lax I., Schlessinger, J. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. Mol. and Cell. Biol., 18:3966-3973, 1998.
6. Larsson, H., Klint, P., Landgreen, E., Claesson-Welsh, L. Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk. J. of Biol. Chem., 274:25736-25734, 1999.
7. Lim, Y.P., Low, B.C., Lim, J., Wong, S.M., Guy, G.R. Association o atypical protein kinase C isotypes with the docker protein FRS2 in fibroblast growth factor signaling. J. of Biol. Chem., 274:19025-19034, 1999.
8. Kurebayashi, J., McLeskey, S.W., Johnson, M.D., Lippman, M.E., Dickson, R.B., Kern F.G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. Cancer Research, 53:2178-2187, 1993.

9. Klint, P., Kanda, S., Claesson-Welsh, L. Shc and a novel 89-kDa component couple to the Grb2-Sos complex in fibroblast growth factor-2-stimulated cells. J. of Biol. Chem., 270:23337-23344, 1995.

10. Meakin, S., MacDonald, J., Gryz, E., Kubu, C., Verdi, J. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. J. of Biol. Chem., 274:9861-9870, 1999.

11. Walk, S.F., March, M.E., Ravichandran, K.S. Roles of Lck, Syk, and ZAP-70 tyrosine kinases in TCR-mediated phosphorylation of the adapter protein Shc. Eur. J. Immunol., 28:2265-2275, 1988.

12. Urlinger, S., Baron, U., Thellman, M., Mazahir, T. H., Bujard, H., Hillen, W. Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. PNAS, 97:7963-7968, 2000.

12. Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J., Lax, I. Stimulstion of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. PNAS, 98:6074, 2001

13. Dhalluin, C., Yan, K.S., Plotnikova, O., Lee, K.W., Zeng, L., Kuti, M., Mujtaba, S.,Goldfarb, M., Zhou, M. M. Structural basis of SNT PTB domain interactions with distinct neurotropic receptors. Molecular Cell, 6: 921-929, 2000.

Acronyms and abbreviations.

FGF – fibroblast growth factor

FGFR – fibroblast growth factor receptor

PRF – phenol red free

ER – estrogen receptor

PTB – phosphotyrosine binding domain

CCS – charcoal stripped serum

GFP – green fluorescent protein

MAPK – mitogen-activated protein kinase

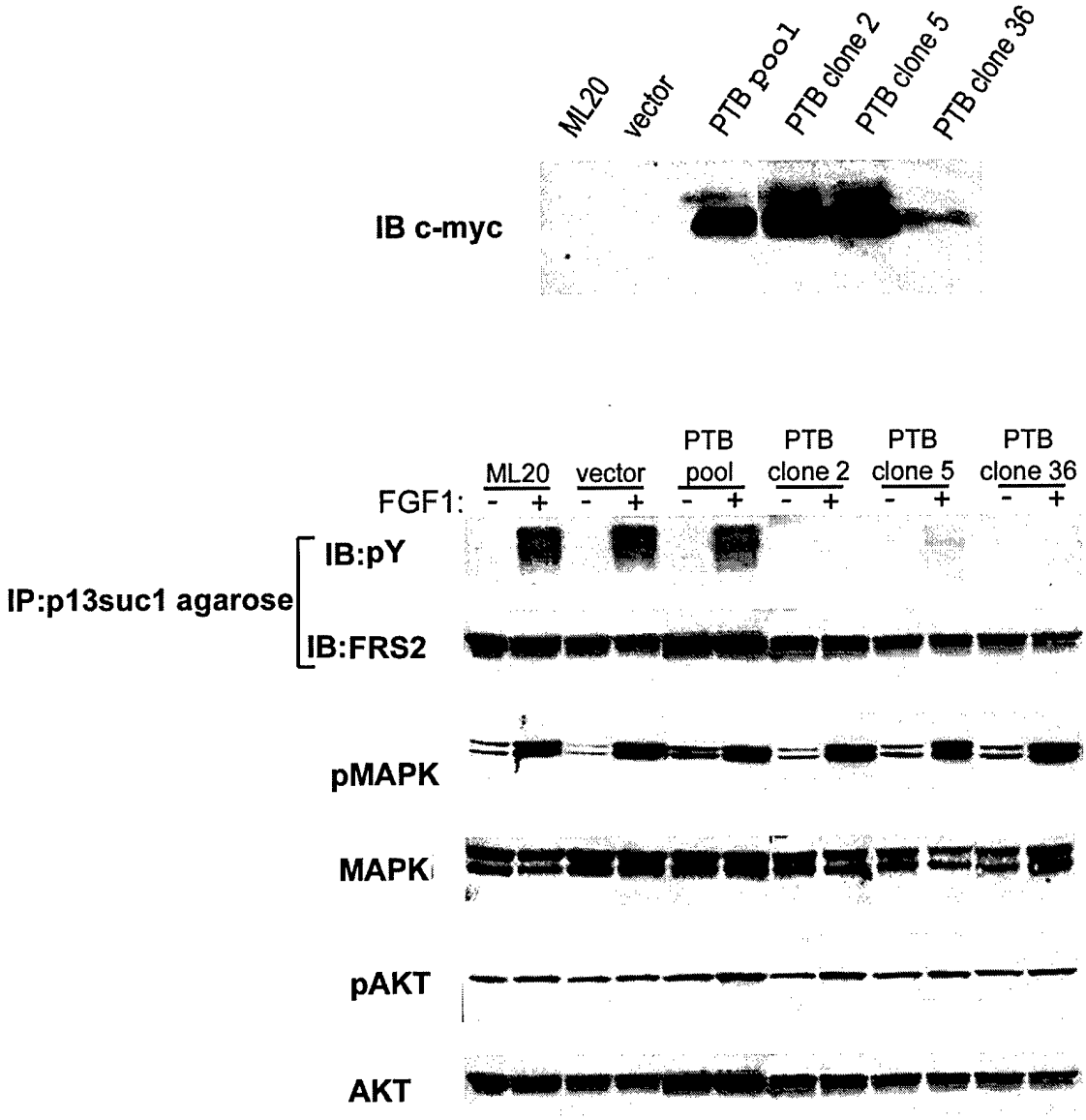
Dox – doxycycline

E2 – 17β estradiol

Appendix

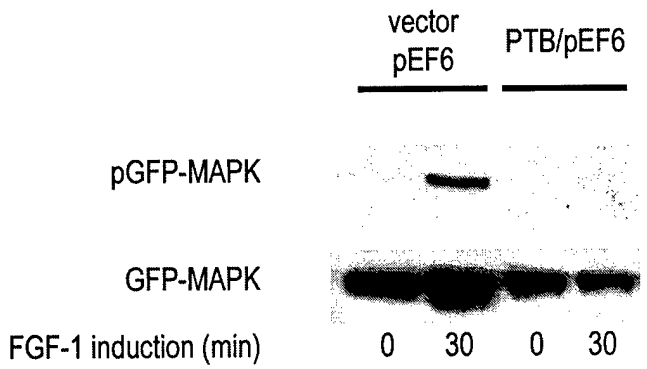
Figures and legends

Fig. 1. Constitutive expression of the SNT-1 PTB domain in MCF-7 cells blocks FGF-1 induced tyrosine phosphorylation of the endogenous SNT-1 protein but fails to reduce FGF induced MAPK or AKT phosphorylation



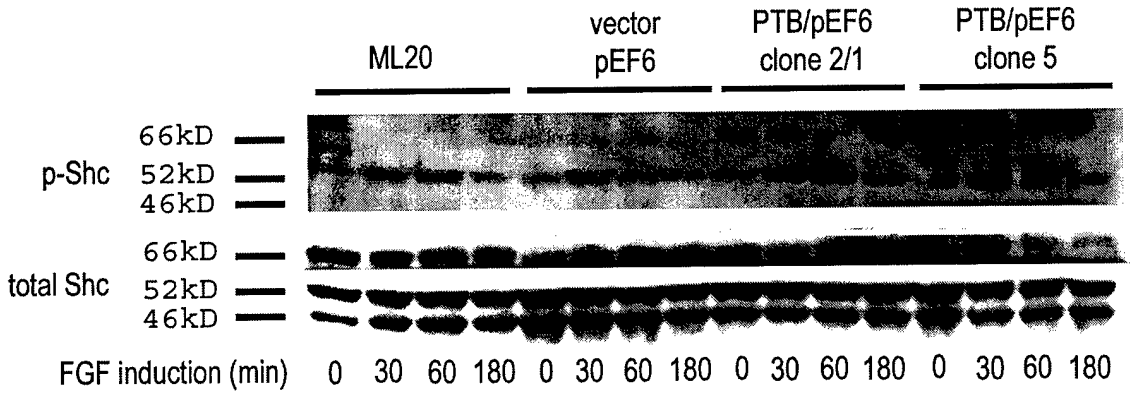
ML20 cells, stably transfected with PTB/pEF6 plasmid, pEF6 vector, or untransfected, were stripped of estrogen in 5% CCS-PRF-IMEM, incubated overnight in serum free PRF-IMEM, and induced with 20 ng/ml FGF-1 and 50 µg/ml heparin in serum free PRF-IMEM for 15 minutes. Total cellular lysates were loaded on 8-16% SDS-PAGE for immunoblotting with monoclonal mouse c-myc antibody 9E10 (Oncogene) (100µg of protein/well), phospho-MAPK and total MAPK antibodies (Cell Signalling) (30µg of protein/well)and phospho-AKT and total AKT antibodies (Cell Signalling) (100µg of protein/well). Tyrosine phosphorylation of the endogenous wild-type SNT-1 is shown in p13Suc1-agarose (Upstate Biotechnology) captured fraction of 1mg of total cellular lysates electrophoresed in 7.5% SDS-PAGE and immunoblotted with 4G10 antiphosphotyrosine antibodies (Upstate).

Fig. 2. Influence of the SNT-1 PTB domain on the phosphorylation of transiently transfected GFP-MAPK in ML20 cells



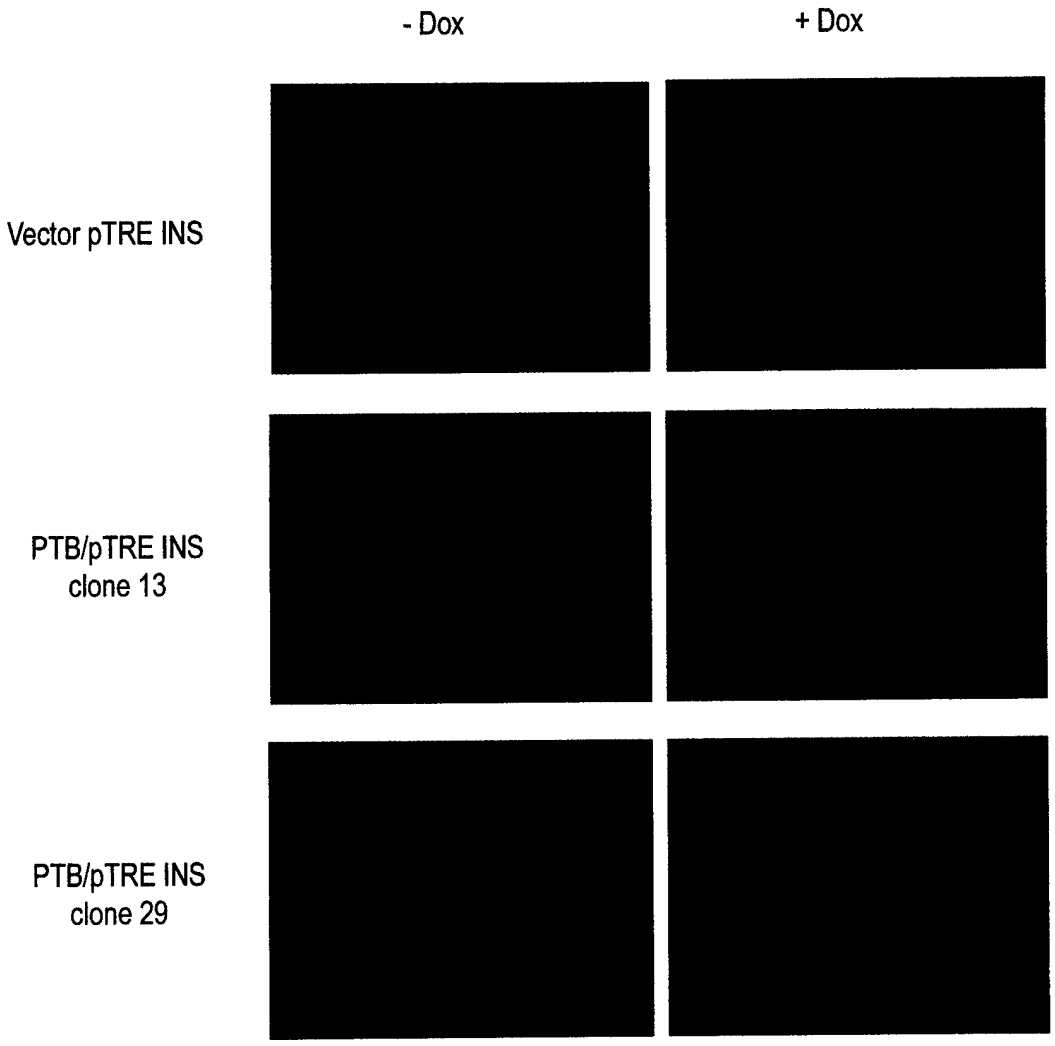
$2x10^5$ ML20 cells in a 60 mm dish were transiently transfected with 0.5 μg GFP-MAPK and 2 μg PTB/pEF6 of GFP-MAPK and pEF6 vector using Lipofectamine Plus (Invitrogen), stripped of estrogen by 3 changes in 5% CCS-PRF-IMEM over 8 hours, incubated overnight in serum free-PRF-IMEM, and incubated with 20 ng/ml FGF-1 and 50 μg/ml heparin in serum free-PRF-IMEM for 30 minutes. 30 μg of total cellular lysate was loaded in each well of 12% SDS-PAGE for immunoblotting with phopho-MAPK and MAPK antibodies.

Fig. 3. Increased FGF-induced phosphorylation of ShcA isoforms in ML20 cells stably transfected with the PTB domain of SNT1 cloned in the pEF6 vector



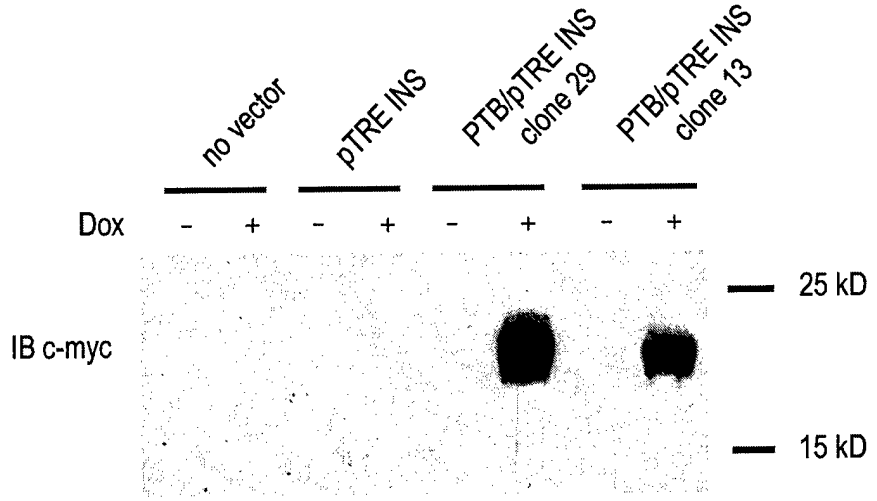
ML20 cells stably transfected with PTB/pEF6 plasmid, pEF6 vector, or untransfected, were treated as described in the legend to Figure 2 and induced with 20 ng/ml FGF-1 and 50 μg/ml heparin in serum free PRF-IMEM for 30, 60, and 180 minutes. 30 μg of total cellular lysate was loaded in each well of 12%SDS-PAGE for immunoblotting with phospho-Shc and total Shc antibodies (Upstate Biotechnology).

Fig. 4. Doxycyline regulated expression of the SNT-1 PTB domain in clones of MCF-7 cells expressing a modified tet-on transactivator



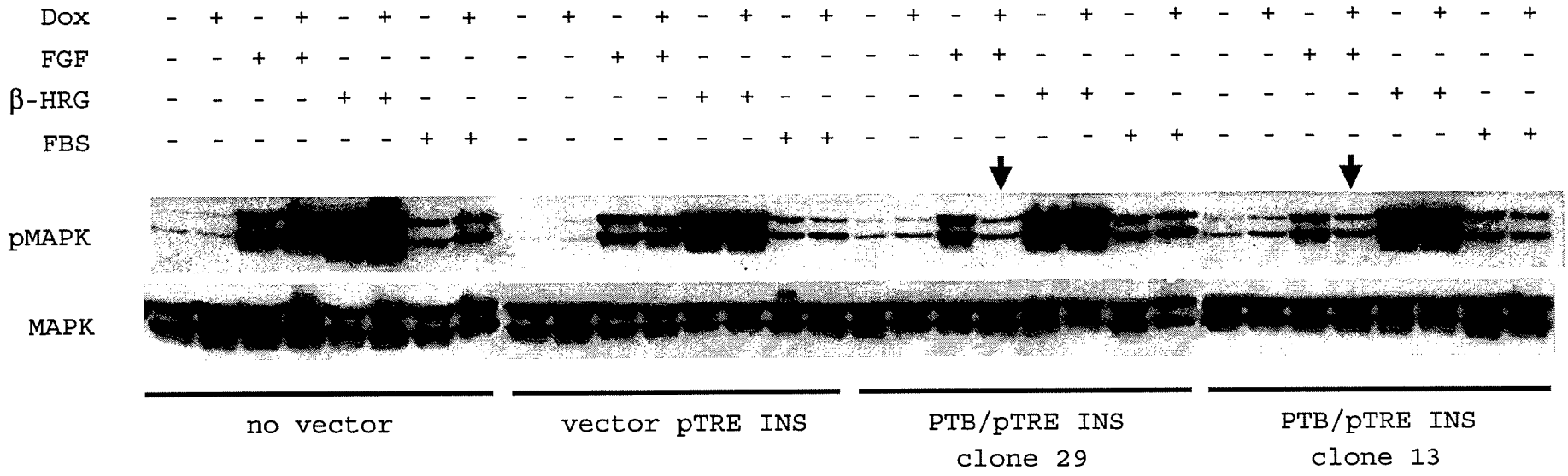
C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 48 hours and visualized with c-myc epitope antibody 4G10 and Texas-red conjugated secondary antibodies.

Fig. 5. Doxycyline regulated expression of the c-myc-tagged SNT-1 PTB domain in ML20 cells



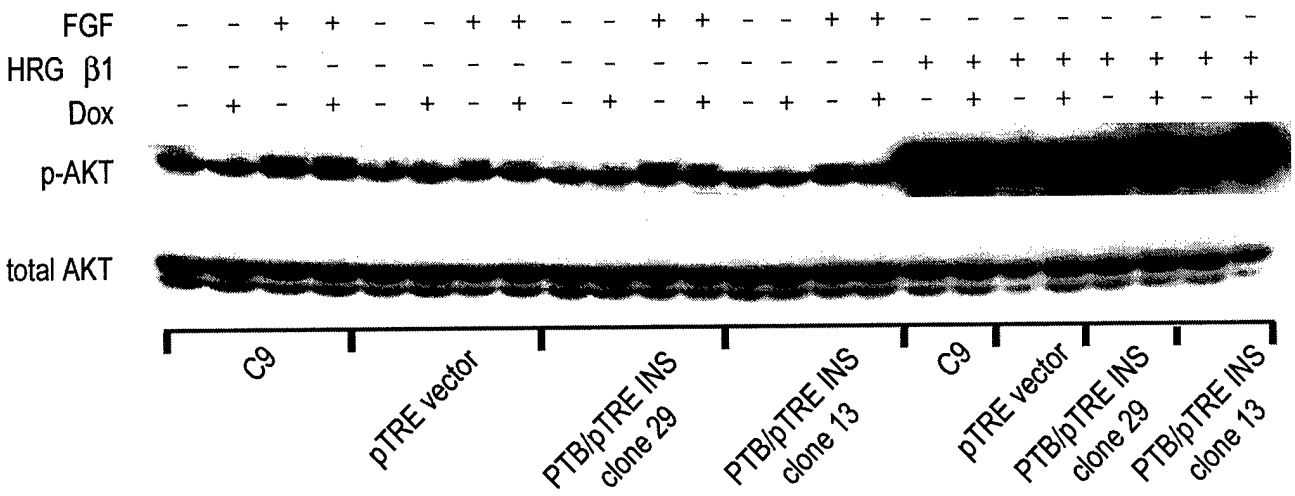
Stably transfected cells were incubated in the presence of 200 ng/ml doxycycline for 48 hours. 5 μg of total cellular lysates were loaded on an 8-16% gradient polyacrylamide gel and used for immunoblotting with the 9E10 mouse monoclonal antibody against the c-myc epitope. Note in the two clones transfected with the pTRE INS vector containing the SNT-1 PTB cDNA insert a high level of induced expression and the absence of basal expression when dox is not present.

Fig. 6. SNT-1 PTB domain specifically reduces FGF-stimulated MAPK phosphorylation when overepressed in a doxycyline regulatable manner



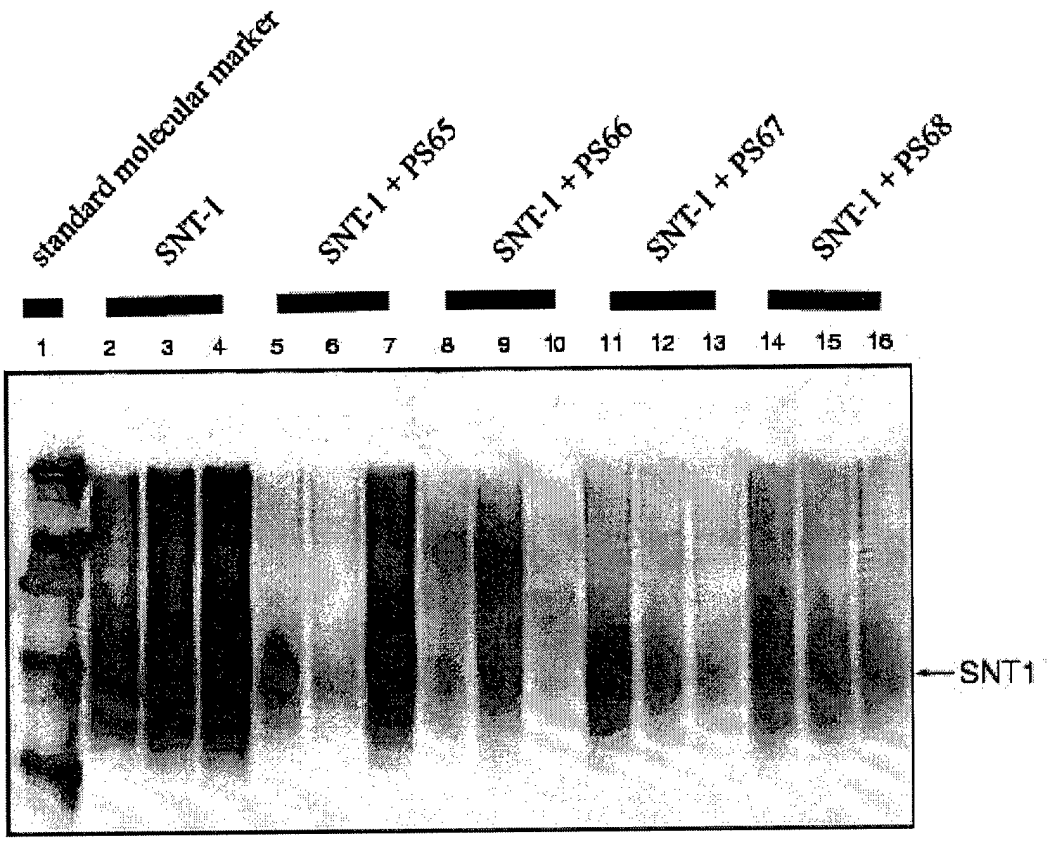
C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 24 hours. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over a 24 hour period and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. MAPK phosphorylation was induced by a 30 minute incubation with 10 ng/ml FGF-1, 10 μg/ml Heregulin β1, or 5% FBS. 30 μg of the total cellular protein was loaded in each well for immunoblotting with phospho-MAPK and MAPK antibodies (Cell Signalling). Note reduced FGF stimulation of MAPK phosphorylation only when SNT-1 PTB is induced (arrows).

Fig. 7. SNT-1 PTB domain does not reduce FGF-stimulated AKT phosphorylation when overexpressed in a doxycycline regulatable manner



C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycyline for 24 hours. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over a 24 hour period and incubated overnight in serum free PRF-IMEM medium. During this process doxycyline remained present at 200 ng/ml where indicated. AKT phosphorylation was induced by a 30 minute incubation with 10 ng/ml FGF-1 or 10 μg/ml Heregulin β-1. 30 μg of the total cellular protein was loaded in each well for immunoblotting with phospho-AKT and AKT antibodies (Cell Signalling).

Fig. 8. Gel Electrophoretic Characterization of SNT1 under Native Conditions



SNT1 protein was mixed with four polypeptides, PS65, PS66, PS67 and PS68 in a ratio of 1:1:5. Samples were mixed with loading buffer containing only 0.5M Tris-HCl, pH 6.8 and 20% glycerol and were run on a 15% acrylamide gel without SDS added in the running buffer (pH 8.3) and stained with Coomassie brilliant blue.